Overexpression of BCL-X₇ Underlies the Molecular Basis for Resistance to Staurosporine-induced Apoptosis in PC-3 Cells

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ABSTRACT

We have reported previously that among human prostate cancer cell lines LNCaP but not PC-3 cells undergo apoptosis after treatment with the protein kinase inhibitor staurosporine (STS). We have now further investigated this model to uncover the molecular mechanism causing resistance to STS-induced apoptosis in PC-3 cells. S-100 lysates of both cell lines showed biochemical changes typical of apoptosis after the addition of cytochrome c and dATP, suggesting that the postmitochondrial phase of apoptosis was intact. Upon addition of STS, the proapoptotic molecules Bax and Bad became predominantly mitochondrial in both cell lines. This, in turn, was followed by loss of mitochondrial transmembrane potential, translocation of cytochrome c to the cytosol, activation of caspase-9, -3, and -7, and cleavage of the apoptotic targets, DNA fragmentation factor and poly(ADP-ribose) polymerase, in LNCaP but not in PC-3 cells. Components of the mitochondrial permeability transition pore, adenine nucleotide transporter and voltage-dependent anion channel, were normally expressed in the correct subcellular fraction of both cell lines. Overexpression of the proapoptotic proteins Bax and Bad, fused to a green fluorescent protein but not of green fluorescent protein alone, induced apoptosis in >80% of PC-3 cells. These experiments suggested that a factor protecting the mitochondria of PC-3 cells mediates resistance to STS-induced apoptosis. A wide search among the antiapoptotic Bcl-2 family members was performed, and Bcl-X₇ was found to be overexpressed in PC-3 cells. Experiments down-regulating Bcl-X₇ expression by using the tyrosine kinase inhibitor genistein, sodium butyrate, or an antisense Bcl-X₇ oligonucleotide restored sensitivity to apoptosis in PC-3 cells. Thus, Bcl-X₇ overexpression is one of the mediators of resistance to STS-induced apoptosis in the prostate cancer cell line PC-3.

INTRODUCTION

Three major apoptotic pathways originating from three separate subcellular compartments have been identified. The receptor-mediated pathway involves the interaction of plasma membrane death receptors with their ligands (1). The mitochondrial pathway involves the functional incapacitation of the mitochondria by proapoptotic Bcl-2 family members (2). Finally, the endoplasmic reticulum pathway (3) is elicited by the application of various forms of stress that result in activation of caspase-12. Although each pathway is initially centered around unique events, the final phase of apoptosis is thought to be common and consists in the activation of the executioner caspases and in their dismantling of substrates critical for cell survival.

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5 The abbreviations used are: STS, staurosporine; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide transporter; DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; RT-PCR, reverse transcription-PCR; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

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major survival pathways active in androgen-independent prostate cancer cells and in other cancer cells resistant to chemotherapy, we performed a careful dissection of the events occurring (and not occurring) in LNCaP and PC-3 cells after STS treatment. Our analysis finds that overexpression of the antiapoptotic factor Bcl-X(2) is one of the mediators of resistance to STS in PC-3 cells in that it protects the mitochondria from undergoing functional incapacitation.

MATERIALS AND METHODS

Materials. Fetal bovine serum and tissue culture media were from Life Technologies, Inc. (Frederick, MD). STS was from Alexis Corporation (San Diego, CA). Genistin was from Research Biochemicals International (Natick, MA). Sodium butyrate was from Sigma Chemical Co. (St. Louis, MO). Antibodies for caspase-3, -7, -Bcl-2, and -Bcl-X were from Transduction Laboratories (Lexington, KY). The anti-cytochrome c, -Bax, and -caspase-9 antibodies were from PharMingen (San Diego, CA). The anti-Bid and DFF antibodies were gifts of Dr. Wang (University of Texas Southwestern, Dallas, TX). The anti-Bad antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-VDAC and ANT antibodies were gifts of Drs. W. Craigen (Baylor College of Medicine, Houston, TX) and H. Schmid (Hormel Institute). The anti-β-actin antibody was from Sigma Chemical Co., and the anti-tubulin was from Chemicon International, Inc. (Temecula, CA). The anti-PARP antibody was from Biomol (Plymouth Meeting, PA). The In Situ Cell Death Detection Kit and the Cellular DNA Fragmentation ELISA kit were from Boehringer Mannheim (Indianapolis, IN). The fluorogenic substrate Z-DEVDAFC was from Enzyme Systems (Dublin, CA). JC-1 was from Molecular Probes (Eugene, OR). RT-PCR was done using the RNA amplification kit from Roche (Branchburg, NJ). The enhanced chemiluminescence detection kit was from Amersham Corp. (Arlington Heights, IL). TUNEL positive cells were scored using a fluorescence microscope (Olympus IX70; Olympus America, Melville, NY). Images were recorded with a digital camera SPOT (Diagnostic Instruments, Sterling Heights, MI). Confocal analysis was performed on an Olympus IMT2 microscope equipped with a Bio-Rad MRC 1024 (Bio-Rad Laboratories, Hercules, CA) scanning apparatus.

Plasmids. Plasmid GFP-Bax was a gift of R. Youle (31). Plasmid GFP-Bad was constructed using a Bad cDNA obtained by PCR from a LNCaP cDNA library. The following primers containing the restriction sites XhoI and XbaI were used: Bad-S, CAAGATCTCTATGCTTCTCACCAGCCAAGAGCT; Bad-AS, CATCTAGATCATCGGGAGGGCGGAGCTTCCCCT. The product of the amplification was cut with the two restriction endonucleases XhoI and XbaI and subcloned in a pEGFP-C1 plasmid (Clontech Laboratories, Inc., Palo Alto, CA) cut with the same enzymes. Sequence analysis was performed to ensure that the two constructs were in frame and that no artifacts were added to the Bad sequence by the amplification process. A VDAC cDNA inserted in the expression plasmid pRc/RSV (Invitrogen, San Diego, CA) was a gift of M. Forte (Vollum Institute, Portland, OR).

Cell Lines and Experimental Design. LNCaP (32) and PC-3 (33) cells have been described previously (5, 27). Two days before the subcellular localization experiments, 1 × 10(6) cells were seeded in a 6-well plate. At the time point 0, cells were treated with 4 μM STS from a 4 mM stock dissolved in DMSO. Adherent and floating cells were recovered 6 h (LNCaP) or 24 h (PC-3) after STS stimulation.

Genistein was given to PC-3 cells at a concentration of 300 μM (from a stock of 300 mM dissolved in DMSO) for 24 h. Vehicle-treated controls were given the same amount of DMSO. Adherent and floating cells were harvested at 24 h after genistein and analyzed for the presence of various markers of apoptosis (see below).

In other experiments, PC-3 cells were treated with sodium butyrate. Cells received 2 mM sodium butyrate (dissolved in water) for 24 or 48 h. Parallel dishes were treated with the same amount of sodium butyrate plus 4 μM STS during the final 24 h of the experiment. Control dishes were treated with vehicle or STS alone. Cells were then collected and studied for various markers of apoptosis (see below).

Cell-free Assay of Apoptosis. As a diagnostic test for the postmitochondrial phase of apoptosis, we used the cell-free assay described by Liu at al. (20). The cell line of interest was harvested and washed twice at 4°C in PBS by centrifugation at 1500 rpm. The resulting pellet was resuspended in one volume of buffer A [HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitors; Sigma Chemical Co.] and incubated at 4°C for 20 min. Cells were then disrupted through passage in a 26-gauge needle 15 times, spun at 1,000 × g at 4°C for 10 min. This supernatant was spun at 100,000 × g for 30 min at 4°C, used for protein determination, aliquoted, and stored at −80°C.

Aliquots of 50 μg of protein were incubated for 30 min at 30°C alone, in the presence of dATP (1 mM), or in presence of both dATP (1 mM) and cytochrome c (10 μM). Aliquots containing 10 μg of protein were then sized in a polyacrylamide gel, and Western analysis for caspase-3, -7, and DFF was performed.

Stable and Transient Transfections. Stable and transient transfections were performed using the LipofectAMINE-plus kit (Life Technologies, Inc.) according to the instructions of the manufacturer, unless stated otherwise. G418 (Life Technologies, Inc.) was used at a final concentration of 400 μg/ml to obtain stable transfectants when necessary.

Western Analysis and Subcellular Fractionation. Western analysis was performed as described previously (5, 27, 30). In each experiment, the same number of μg of cell lysate was loaded, as specified in each case. When precise quantitation was required, densitometric analysis was performed to correct expression of the protein of interest with that of β-actin or tubulin, which were immunodetected in the same sample. Densitometry was done by importing images to a Power Macintosh G3 personal computer using the Chemi Doc Documentation System and the Quantity One quantitation software (both from Bio-Rad, Hercules, CA). Arbitrary densitometric units of the protein of interest were then corrected for the densitometric units of β-actin or tubulin.

Subcellular fractionation was performed using serial centrifugation steps as...
Supernatant (200 μl) were added, and the resulting suspension was gently rocked for 10 min at 4°C and microfuged for 10 min at 4°C. Protein concentration was measured in Co.

### Materials and Methods

**Immunoprecipitation**

Immunoprecipitation was performed according to Gross et al. (34, 35) with some modifications. Briefly, LNCaP and PC-3 cells were washed twice in ice-cold PBS, resuspended in five volumes of extraction buffer [containing 220 mm mannitol, 68 mm sucrose, 50 mm PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM DTT, and protease inhibitors; Sigma Chemical Co.; added at 1:100 dilution], and kept on ice for 15 min. Cells were then spun at 400 g for 10 min at 4°C to collect the heavy membrane, mitochondrial-enriched pellet. This supernatant was centrifuged at 10,000 × g for 10 min at 4°C to collect the heavy membrane, mitochondrial-enriched pellet. The supernatant was then spun at 100,000 × g for 30 min at 4°C to separate the light membrane ER-enriched pellet (not used in these experiments) from the supernatant (containing the cytosol). Pilot experiments demonstrated the ability of this technique to yield subcellular fractions enriched with mitochondria or cytosol. For instance, cytochrome c was recovered uniquely from the mitochondrial fraction in cells not undergoing apoptosis and from both the cytosolic and mitochondrial fractions when cells were undergoing apoptosis. In contrast, the proteins VDAC and ANT (located on the outer and inner mitochondrial membrane, respectively) were recovered uniquely from the mitochondrial fraction, regardless of whether the cells were undergoing apoptosis or not.

**Transfection**

Subcellular localization of VDAC in PC-3 and LNCaP cells. A and B, 10 μg of cell lysates obtained from each subcellular fraction were sized and processed as described in “Materials and Methods.” A representative experiment of three is shown.

**Immunofluorescence**

Immunofluorescence of Bcl-xL and Bax was performed using PC-3 and LNCaP cells grown on coverslips. After 48 h, cells were washed, fixed with paraformaldehyde 3.7% in PBS for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 for 5 min. Cells were then incubated with anti-Bax (mouse monoclonal; Santa Cruz Biotechnology) and anti-Bcl-xL (goat polyclonal; Santa Cruz Biotechnology) antibodies for 60 min at room temperature. After extensive washes in PBS, cells were stained with 568 Alexa Fluor-conjugated antigen antibody and 488 Alexa-conjugated antimouse antibody (Molecular Probes). Nuclei were counterstained with TO-PRO 3 (Molecular Probes). Cells were examined using an Olympus IMT-2 microscope equipped with an MRC-1024 laserscan confocal system (Bio-Rad) using the 488-nm, 568-nm, and 647-nm excitation lines from an Argo-krypton laser. Green (Bax), red (Bcl-xL), and ultra-red emissions were collected and processed with the Lasersharp 1024 software (Bio-Rad).

### Results

**Identification of Apoptotic Cells after Transient Transfection of PC-3 Cells with GFP-Bad and GFP-Bax**

PC-3 cells growing on coverslips were transiently transfected with GFP, GFP-Bad, and GFP-Bax using the calcium precipitation kit from 5 prime-3 prime, Inc. (Boulder, CO). After 12 h, cells were fixed for 30 min at 4°C in 4% formaldehyde dissolved in PEM buffer [400 mM Potassium PIPES, 0.5 mM EGTA (pH 7.0), 1 mM MgCl2], washed with PEM buffer, treated in 0.1 mM NH4Cl (to quench autofluorescence), incubated for 30 min at room temperature in PEM + 0.5% Triton X-100, and washed again in PEM × 3. 4’,6-Diamidino-2-phenylindole dihydrochloride (Molecular Probes) staining was then performed using a 1 mg/ml stock diluted 2000-fold for 1 min. After mounting the coverslip, cells were observed under a fluorescence microscope. Cells showing green fluorescence were then ob-

![Image](cancerres.aacrjournals.org)
Bcl-X<sub>L</sub> was evaluated using the quantitative Western analysis described above. OptiMEM). Cells were harvested 48 h after transfection, and the expression of Bcl-X<sub>L</sub> was performed to control its down-regulation. The other dishes were treated with 4 μM STS for 24 h. The presence of apoptosis was verified using the Cellular DNA Fragmentation ELISA kit from Boehringer.

RESULTS

To evaluate the integrity of the postmitochondrial machinery, cytosols from PC-3 and LNCaP cells were incubated in the presence and absence of bovine heart cytochrome c and dATP using the cell-free assay of Liu et al. (20). When all of these components were present, several hallmarksof apoptosis were evident, including cleavage of caspase-3, caspase-7, and DFF (Fig. 1). These results indicated that the postmitochondrial phase of the mitochondrial pathway of apoptosis was intact and functional in the two cell lines and that the locus of resistance to STS-induced apoptosis in PC-3 cells was at the primi-

Intracellular Trafficking of Apoptotic Signaling Molecules. Because the subcellular localization and proteolytic processing of members of the Bcl-2 family, the caspase family, and cytochrome c are critical to regulating proper engagement of the apoptotic machinery, we evaluated the potential role of these molecules in mediating apoptotic responses. In LNCaP cells (Fig. 2), the proapoptotic Bid was in the cytoplasm in the basal state and nearly completely proteolyzed in response to STS, most likely after caspase-dependent cleavage at one of its aspartate residues (40, 41). In PC-3 cells, Bid was unaffected by STS. In both lines, Bad and Bax translocated from the cytoplasm to the mitochondria with STS. Although Bcl-X<sub>L</sub> was insensitive to STS in both lines, it was almost exclusively mitochondrial in LNCaP cells but equally distributed in PC-3 mitochondria and cytoplasm. As we reported previously (5, 27), cytochrome c translocated from the mitochondria to the cytoplasm in LNCaP cells, leading to cleavage of caspsases-9, -3, and -7, independent of their subcellular localization. Conversely, these postmitochondrial mediators were STS-insensitive in PC-3 cells.

Molecular Analysis of STS Resistance in PC-3 Cells. On the basis of the above data, we deduced that the locus of resistance to STS was at the mitochondrial phase. Sequence analysis of Bax and Bad demonstrated that both these molecules were wild type in PC-3 cells. The only abnormality consisted in a polymorphism-changing codon 241 of Bad from CCT to CCA (Pro → Pro). Thus, to identify the
precise molecular defect, we investigated the expression and functional integrity of the VDAC (42) and the ANT (43), key mediators of the mitochondrial permeability transition pore. In both cell lines, VDAC (Fig. 3, A and B) and ANT (data not shown) were of the appropriate molecular weight and uniquely localized to the mitochondria (Fig. 3, A and B). VDAC coimmunoprecipitated with Bax and Bad, indicating normal functional interactions (Fig. 3C). Further, overexpression of VDAC had no effect on PC-3 cell sensitivity to STS (data not shown).

Overexpression of Bad and Bax Induces Apoptosis in PC-3 Cells. Next, we overexpressed GFP-Bax and -Bad to determine whether the mitochondrial phase of PC-3 cells was actually intact and could be forcibly engaged or if some other defect existed. Cells showing GFP fluorescence were scored as normal or apoptotic based on their morphology (Fig. 3A) and were found to be apoptotic in 90 and 92% of the cases after transfection with either GFP-Bax or GFP-Bad (Fig. 4). In contrast, only 6% of the cells transfected with the control plasmid expressing only the GFP protein were apoptotic (Fig. 4). These results confirmed that in PC-3 cells the mitochondrial apoptotic program could be activated if properly stimulated by proapoptotic Bcl-2 family members. In addition, these data suggested that the components of the mitochondrial permeability transition pore were able to mediate mitochondrial incapacitation after the interaction with proapoptotic Bcl-2 family members such as Bax and Bad. Subsequently, a systematic analysis of Bcl-XL, Bcl-2, Bcl-w, mcl-1, and A1, molecules known to prevent apoptosis by protecting the mitochondria, was initiated in several prostate cancer cell lines. Most notably, Bcl-XL expression was 45-fold greater in PC-3 compared with LNCaP cells (Fig. 5A). Overexpression of Bcl-XL was also confirmed in experiments of immunofluorescence as shown in Fig. 5, B and C, where Bcl-XL (red fluorescence) is significantly more expressed in PC-3 (Fig. 5C) compared with LNCaP cells (Fig. 5B).

Down-Regulation of Bcl-XL Restores Sensitivity to STS-induced Apoptosis in PC-3 Cells. Genistein, a nonspecific inhibitor of protein tyrosine kinases, was reported previously (44) to down-regulate Bcl-XL expression. In PC-3 cells, whereas STS actually increased Bcl-XL about 2-fold, genistein caused a 2.3-fold decrease (Fig. 6). Further, genistein alone induced apoptosis by causing loss of mitochondrial transmembrane potential, release of cytochrome c to the cytosol, activation of the caspase pathway, and cleavage of the caspase target PARP (Fig. 7).

Butyric acid, which also was reported to down-regulate Bcl-XL expression (45), caused a 7.3-fold reduction in Bcl-XL protein (Fig. 8A). However, this was associated with only minimal cytochrome c translocation to the cytosol (compare Fig. 8B [baseline] versus Fig. 8C [after 48 h of butyric acid]) and minor cleavage of caspase-9 and caspase-7 or DFF (Fig. 8E). Nonetheless, significant cleavage of caspase-3 (Fig. 8E) was accompanied by a 15-fold induction of DEVDase (data not shown) and 37% TUNEL positivity (Fig. 8, H and I). Most importantly, butyric acid treatment conferred STS sensitivity to PC-3 cells as evidenced by additional down-regulation of Bcl-XL (Fig. 8A), cytochrome c translocation from mitochondria to cytoplasm (compare Fig. 8B [baseline] with Fig. 8D [after 48 h of butyric acid + STS from 24 to 48 h]), complete cleavage of caspases-9, -3, and -7 as well as DFF (Fig. 8E), 18.5-fold increased DEVDase activity (data not shown), and massive (96%) induction of TUNEL positivity (Fig. 8, F and G).

Finally, antisense oligonucleotides were used to decrease Bcl-XL expression in PC-3 cells. Antisense treatment for 24 h decreased Bcl-XL protein ~4-fold compared with a mismatched control oligo and resulted in a 6-fold increase in apoptosis (Fig. 9).

DISCUSSION

The data presented here illustrate clear molecular differences in the susceptibility of different prostate cancer cell lines to undergo apoptosis. Whereas LNCaP cells engaged many of the typical components of the apoptotic machinery, PC-3 cells were extremely resistant to apoptosis. Using a cell-free system to reconstitute the postmitochondrial phase, it was evident that the two lines were equally capable of...
normal and suggested that an antiapoptotic factor with the ability to protect the mitochondria was overexpressed in PC-3 cells. Four lines of evidence support the hypothesis that Bcl-X<sub>L</sub> overexpression is responsible for the resistance of the PC-3 cells to STS-induced apoptosis: a) Bcl-X<sub>L</sub> was overexpressed in PC-3 cells relative to several apoptosis-sensitive prostate cell lines; b) genistein, a tyrosine kinase inhibitor, down-regulated Bcl-X<sub>L</sub> and induced spontaneous apoptosis; c) sodium butyrate down-regulated Bcl-X<sub>L</sub> expression and, most importantly, conferred STS sensitivity; and d) antisense down-regulation of Bcl-X<sub>L</sub> restored STS-induced apoptosis. These results indicated that elevated Bcl-X<sub>L</sub> was responsible for protecting PC-3 cells from apoptosis.

Overexpression of Bcl-X<sub>L</sub> has already been described in PC-3 cells (46) and in cell lines manifesting multiple drug resistance (47, 48). In the latter, overexpression of Bcl-X<sub>L</sub> is associated with apoptosis resistance because of abrogation of cytochrome c release to the cytosol. Bcl-X<sub>L</sub> overexpression does not functionally substitute for a mutagenic initiator or mitogenic promoter in tumorigenesis. However, there is an increased potential for benign tumors overexpressing Bcl-X<sub>L</sub> to undergo malignant degeneration possibly because of their prolonged survival (49). The mechanism leading to apoptosis resistance after Bcl-X<sub>L</sub> overexpression has to do with its ability to prevent cytochrome c translocation to the cytosol and to protect the mitochondria from undergoing functional incapacitation (50). This is in part achieved through inactivation of proapoptotic Bcl-2 family members via heterodimerization (51, 52) and in part by facilitating mitochondrial ATP/ADP exchange (53).

The signaling pathway regulating Bcl-X<sub>L</sub> expression is only partially understood. Using murine myeloid cell lines, Bcl-X<sub>L</sub> expression was found to be positively regulated by IFN-γ (54) and through a Jak...
kinase-dependent interleukin 3 pathway (55). Other investigators have identified a connection between induction of Bcl-X \(_L\) expression and the activity of tyrosine kinase receptors such as HER2 (13, 56) and a tumor-specific mutant of epidermal growth factor receptor (57). The fact that the tyrosine kinase inhibitor, genistein, decreased Bcl-X \(_L\) expression in PC-3 cells supports the conclusion that overexpression of Bcl-X \(_L\) could be mediated through this signaling pathway. Another mechanism of Bcl-X \(_L\) regulation is by inactivation through posttranslational modifications. For instance, in response to genotoxic agents, the stress-activated protein kinase translocates to the mitochondria and phosphorylates Bcl-X \(_L\), presumably leading to its inactivation (58).

Upon demonstrating Bcl-X \(_L\) overexpression in PC-3 cells, we wondered if this is a common occurrence in other prostate cancer cell lines or in clinical specimens from patients with prostate cancer. Other prostate cancer cell lines such as DU-145 and TSU-Pr (1) express higher concentrations of Bcl-X \(_L\) than LNCaP, although not to the same extent as PC-3 (Fig. 5). Tissue from patients with prostate cancer has not been investigated for its presence of Bcl-X \(_L\) overexpression, despite the fact that since 1992 an extensive literature has accumulated on new potential therapeutic targets to decrease the threshold of apoptosis. For instance, in response to genotoxic agents, higher concentrations of Bcl-X \(_L\) than LNCaP, although not to the same extent as PC-3 (Fig. 5). Tissue from patients with prostate cancer has not been investigated for its presence of Bcl-X \(_L\) overexpression, despite the fact that since 1992 an extensive literature has accumulated on new potential therapeutic targets to decrease the threshold of apoptosis.

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